



# SPECIES DISTINCTNESS OF *BITHYNIA CETTINENSIS* CLESSIN, 1887 AND *B. ZETA* GLÖER ET PEŠIĆ, 2007 (CAENOCASTROPODA: TRUNCATELLOIDEA)

VLADIMIR PEŠIĆ<sup>1</sup>, SEBASTIAN HOFMAN<sup>2</sup>, ALEKSANDRA RYSIEWSKA<sup>3</sup>, ARTUR OSIKOWSKI<sup>4</sup>,  
ANDRZEJ FALNIOWSKI<sup>3\*</sup>


<sup>1</sup>Department of Biology, Faculty of Sciences, University of Montenegro, Cetinjski put b.b.,  
81000 Podgorica, Montenegro

<sup>2</sup>Department of Comparative Anatomy, Institute of Zoology and Biomedical Research, Jagiellonian  
University, Gronostajowa 9, 30-387 Kraków, Poland (e-mail: [s.hofman@uj.edu.pl](mailto:s.hofman@uj.edu.pl));

 <https://orcid.org/0000-0001-6044-3055>

<sup>3</sup>Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University,  
Gronostajowa 9, 30-387 Kraków, Poland (e-mails: [a.rysiewska@uj.edu.pl](mailto:a.rysiewska@uj.edu.pl); [andrzej.falniowski@uj.edu.pl](mailto:andrzej.falniowski@uj.edu.pl));  
AR  <https://orcid.org/0000-0002-9395-9696>, AF  <https://orcid.org/0000-0002-3899-6857>

<sup>4</sup>Department of Animal Anatomy, Institute of Veterinary Science, University of Agriculture in Krakow,  
Mickiewicza 24/28, 30-059 Kraków, Poland (e-mail: [a.osikowski@urk.edu.pl](mailto:a.osikowski@urk.edu.pl));

 <https://orcid.org/0000-0001-6646-2687>

\*corresponding author

**ABSTRACT:** Shells of *Bithynia*: widely distributed *B. tentaculata* (Linnaeus), Balkan *B. cettinensis* Clessin from Cetina River in Croatia, and *B. zeta* Glöer et Pešić from Vitoja spring at Lake Skadar, Montenegro, as well as male genitalia of *B. zeta* and *B. cettinensis* were examined. The shells of all three taxa are similar, also the penes differ only slightly. The mean value of the length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin, was 1.63 for *B. zeta*, and 1.33 for *B. cettinensis*. Those values differed slightly, especially compared to 5.0–5.9 for *B. tentaculata*. In the maximum likelihood (ML), as well as Bayesian (BI) trees, *B. cettinensis* and *B. tentaculata* were sister clades with p-distance of 0.007, and *B. zeta* was more distinct, with p-distance of 0.122 to *B. cettinensis* and 0.154 to *B. tentaculata*. The species distinctness of the three studied taxa was confirmed.

**KEY WORDS:** species distinctness, shell, penis, tubular penial gland, mtDNA, COI

## INTRODUCTION

The Bithyniidae Gray, 1857 inhabit fresh and brackish waters of Europe, Asia Minor, northern Asia, India, Indochina, China, part of Australia, western Africa, Lake Tanganyika, and some introduced localities in North America (FALNIOWSKI 1989). Apart from the “normal” feeding on detritus, they are also filter-feeders (LILLY 1953, FRETTER & GRAHAM 1962). *Bithynia tentaculata* (Linnaeus, 1758) is one of the most widely distributed and most common freshwater species. Some other nominal species of *Bithynia* Leach, 1818, whose distinctness remains unclear, are known from southern Europe. CLESSIN (1887) described *B. cettinensis* Clessin, 1887, from Cetina River in Croatia.

GLÖER & PEŠIĆ (2007) described three new species of *Bithynia* from Lake Skadar in Montenegro: *B. zeta* Glöer et Pešić, 2007, *B. skadarskii* Glöer et Pešić, 2007, and *B. radomani* Glöer et Pešić, 2007. The penis and penial gland morphology presented by GLÖER & BERAN (2009) for *B. cettinensis* was based on only one penis, most probably somewhat damaged, and molecular data for the European *Bithynia* are known only for *B. tentaculata*. The aim of the present paper was to describe the penial morphology of *B. cettinensis* and to check the species distinctness of *B. tentaculata*, *B. cettinensis*, and *B. zeta*, applying molecular data, namely cytochrome oxidase subunit I (COI) sequences.



## MATERIAL AND METHODS

We collected specimens of the three *Bithynia* species: *B. zeta* from Vitoja spring (42°19'32"N, 19°21'46"E; Figs 1–2), Montenegro, *B. cettinensis* from Cetina River at Radmanove Mlinice (43°26'37"N, 16°45'02"E), Croatia, and *B. tentaculata* from pond in Spytkowice near Kraków (50°00'14"N, 19°29'10"E), Poland. The morphology of *B. tentaculata* was studied in many localities throughout Poland (FALNIOWSKI 1989); shells from Lake Gardno (N. Poland) are shown in the present paper.

For molecular study, the snails were washed twice in 80% ethanol and left to stand in it for ca. 12 hours. Afterwards, the ethanol was changed twice in 24 hours and finally, after a few days, the 80% solution was exchanged for a 96% one, stored at –20 °C.

The shells were photographed with a CANON EOS 50D digital camera, under a NIKON SMZ18

microscope with dark field. Dissection was done under NIKON SMZ18 microscope, and photographed with dark field. The measurements of the penis and tubular penial gland were taken on the photographs, with SILVA Map Measurer Plus curvimeter. The morphological terminology follows HERSHLER & PONDER (1998).

The tissue was hydrated in TE buffer (3 × 10 min.); then total genomic DNA was extracted with the SHERLOCK extracting kit (A&A Biotechnology), and the final product was dissolved in 20 µl TE buffer. The extracted DNA was stored at –80 °C at the Department of Malacology, Institute of Zoology and Biomedical Research, Jagiellonian University in Kraków (Poland). Details of PCR conditions, primers used and sequencing were given in SZAROWSKA et al. (2014). Cytochrome oxidase subunit I (COI) mtDNA

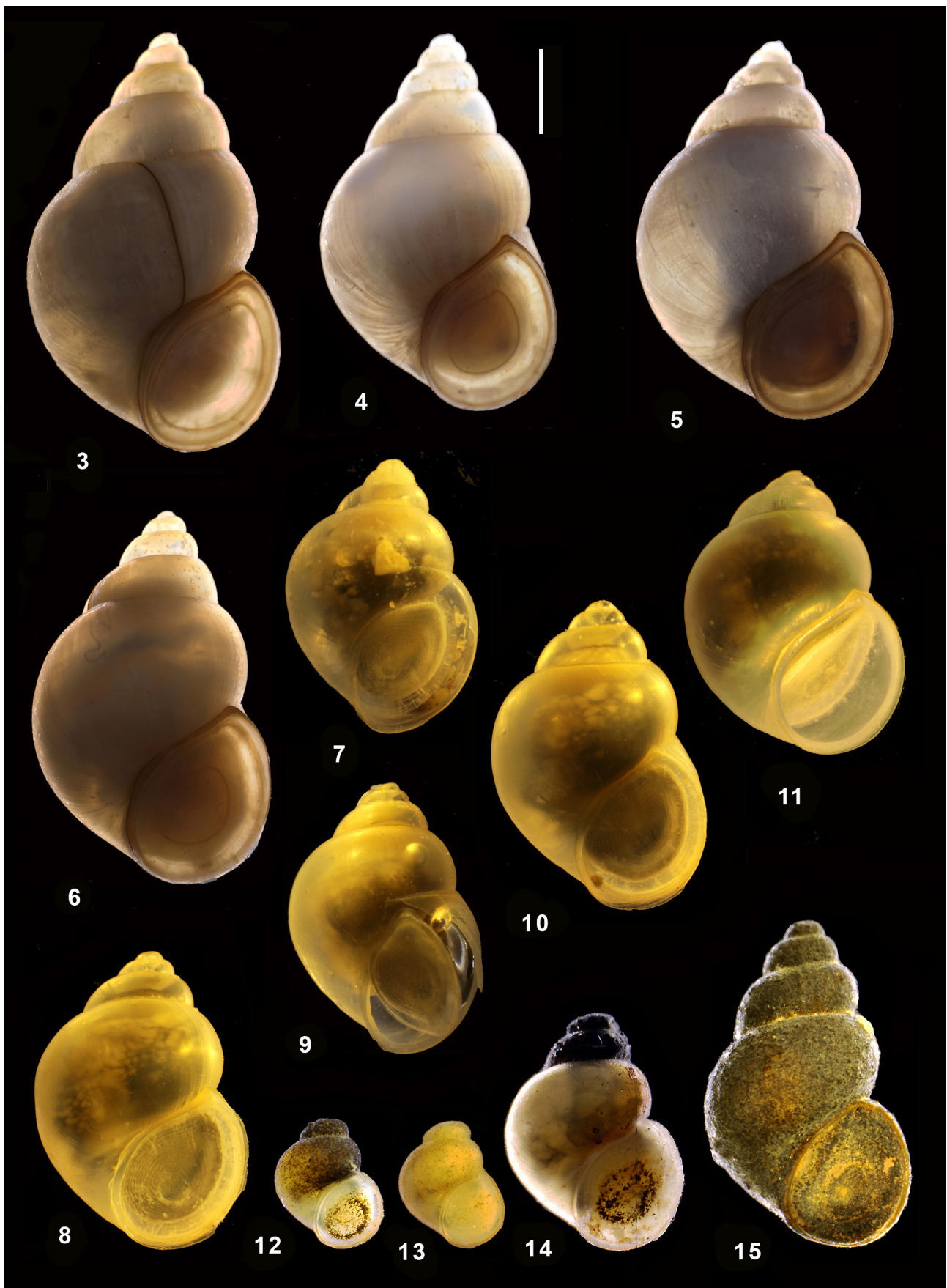


Figs 1–2. Vitoja Spring

Table 1. Taxa used for phylogenetic analyses with their GenBank accession numbers and references

Species	COI GB numbers	References
<i>Bithynia tentaculata</i> (Linnaeus, 1758)	MN013036–MN013037	present study
<i>Bithynia zeta</i> Glöer et Pešić, 2007	MN013038–MN013041	present study
<i>Bithynia cettinensis</i> Clessin, 1887	MN013042	present study
<i>Bithynia</i> sp. Leach, 1818 – Egridir Lake, Turkey	FJ160291	BENKE et al. 2009
<i>Bithynia</i> sp. Leach, 1818 – Poso Lake, Indonesia	KY574006	VAN BOCXLAER et al. 2017
<i>Bithynia funiculata</i> Walker, 1927	KY118598	KULSANTIWONG et al. 2013
	KY118601	KULSANTIWONG et al. 2013
<i>Bithynia siamensis goniomphalos</i> (Morelet, 1866)	KY118603	KULSANTIWONG et al. 2013
	KY118610	KULSANTIWONG et al. 2013
	KY118619	KULSANTIWONG et al. 2013
<i>Bithynia siamensis siamensis</i> (Lea, 1856)	KY118658	KULSANTIWONG et al. 2013
<i>Bythinella austriaca</i> (von Frauenfeld, 1857)	JQ639858	FALNIOWSKI et al. 2012
<i>Bythinella micherdzinskii</i> Falniowski, 1980	JQ639854	FALNIOWSKI et al. 2012
<i>Gabbia pygmaea</i> (Preston, 1908)	KY118682	KULSANTIWONG et al. 2013
<i>Gabbia wykoffi</i> (Brandt, 1968)	KY118724	KULSANTIWONG et al. 2013
<i>Hydrobioides nassa</i> (Theobald, 1865)	KY118756	KULSANTIWONG et al. 2013
	KY118765	KULSANTIWONG et al. 2013
<i>Wattebledia baschi</i> Brandt, 1968	KY118769	KULSANTIWONG et al. 2013
<i>Wattebledia crosseana</i> (Wattebled, 1884)	KY118793	KULSANTIWONG et al. 2013





Figs 3–15. Shells of the studied *Bithynia*: 3–6 – *B. tentaculata*, Lake Gardno, Poland; 7–11 – *B. cettinensis*, Radmanove Mlinice, Cetina River, Croatia; 12–15 – *B. zeta*, Vitoja Spring, Montenegro. Scale bar 2 mm



was amplified with the following primers: LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (FOLMER *et al.* 1994), and COR722b 5'-TAAACTTCAG GGTGACCAAAAAATYA-3' (WILKE & DAVIS 2000). PCR conditions for the COI were as follows: initial denaturation step of 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension of 4 min at 72 °C. Sequencing methods are described in SZAROWSKA *et al.* (2014).

Sequences were initially aligned in the MUSCLE (EDGAR 2004) programme in MEGA 6 (TAMURA *et al.* 2013) and then checked in Bioedit 7.1.3.0 (HALL 1999). The saturation test (XIA 2000, XIA *et al.* 2003)

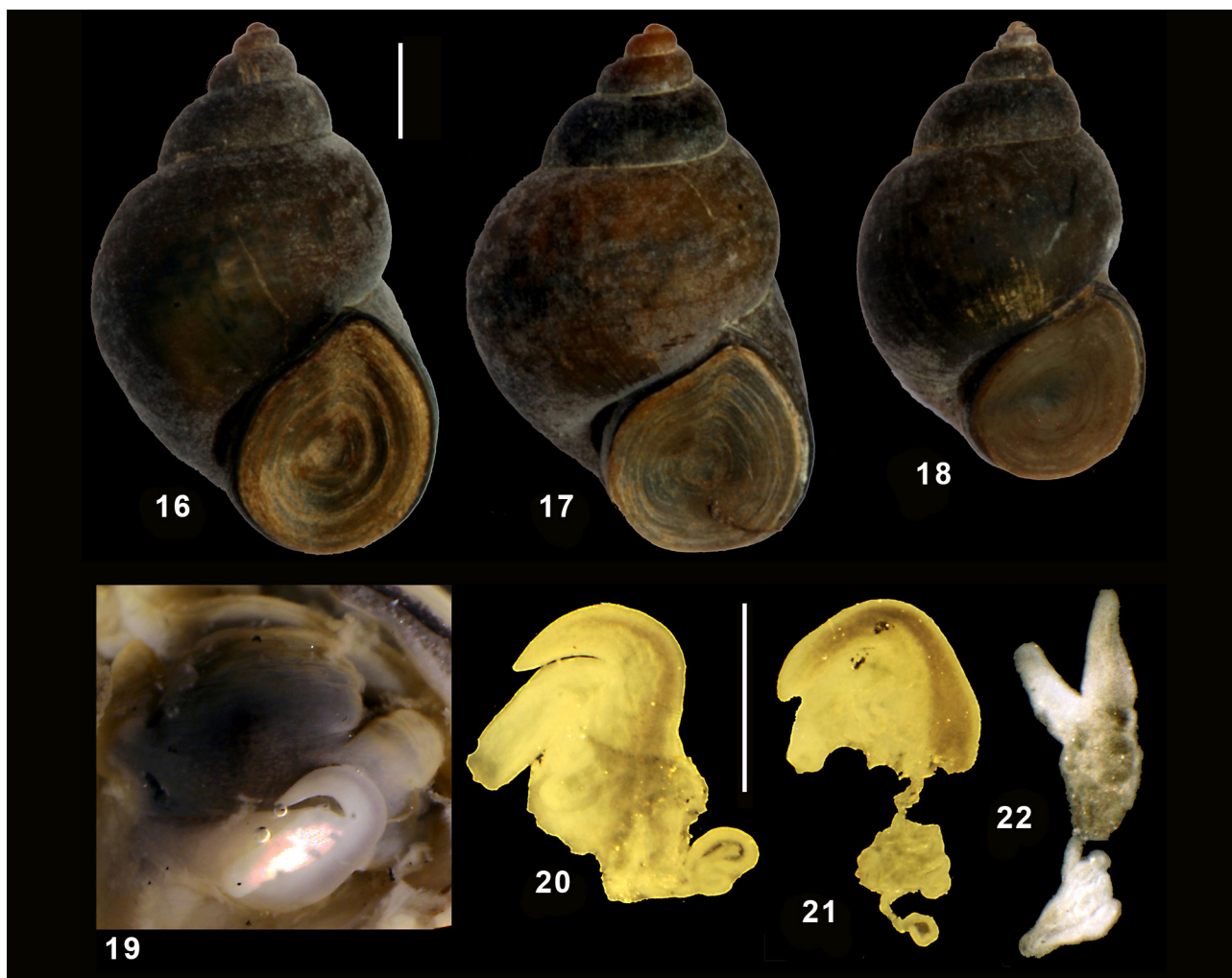
was performed using DAMBE (XIA 2013). In the phylogenetic analysis additional sequences from GenBank were used (Table 1). The data were analysed using approaches based on Bayesian inference (BI) and maximum likelihood (ML). We applied the GTR model, whose parameters were estimated by the RaxML (STAMATAKIS 2014). The BI analyses were run using MrBayes v. 3.2.3 (RONQUIST *et al.* 2012) with the default priors. The ML approach was applied with RaxML v. 8.0.24 (STAMATAKIS 2014). RaxML analyses were performed using the free computational resource CIPRES Science Gateway (MILLER *et al.* 2010).

## RESULTS

### MORPHOLOGY

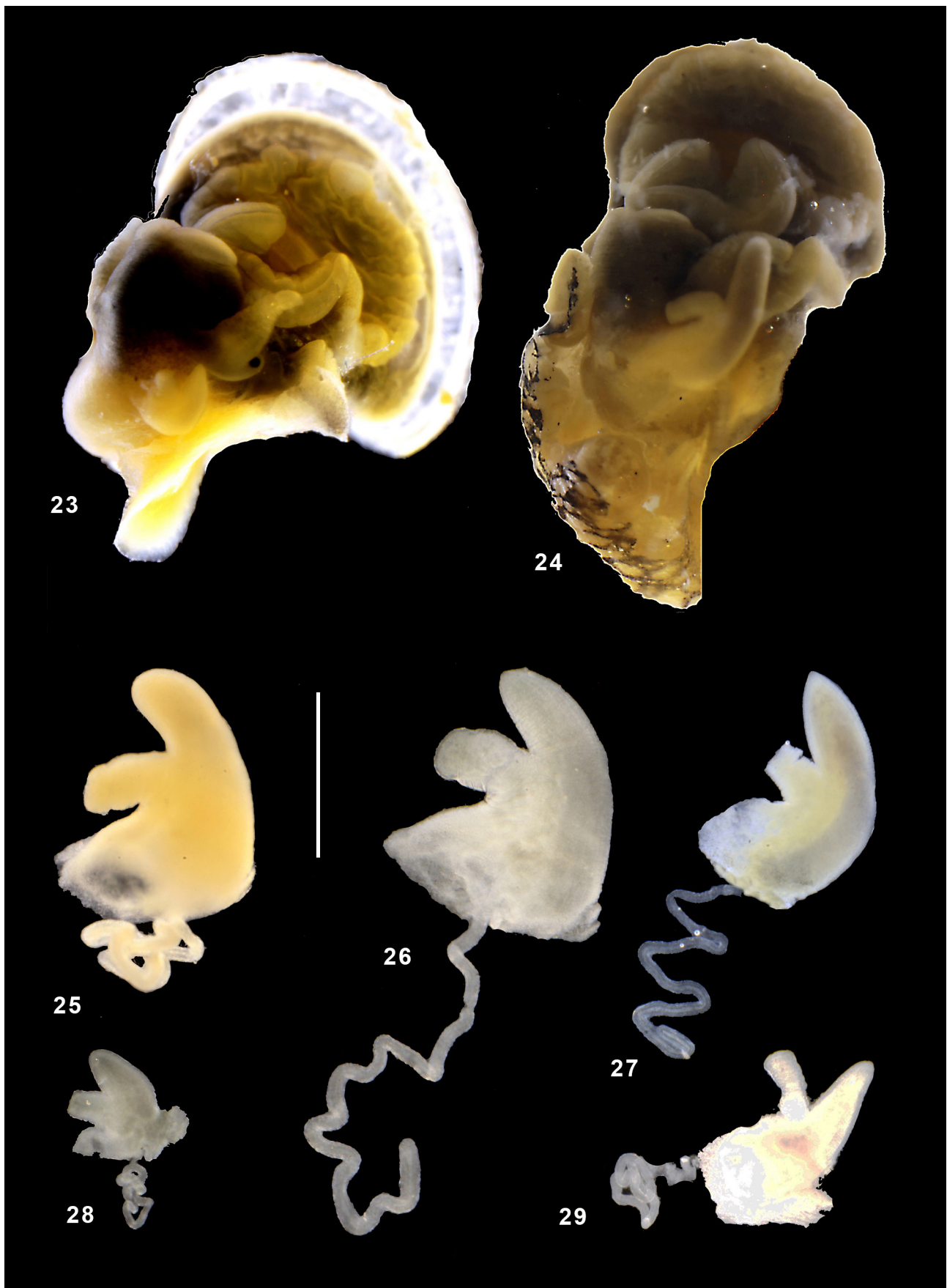
The shells of *Bithynia tentaculata* (Figs 3–6) and *B. cettinensis* (Figs 7–11) are slightly variable and similar: only smaller dimensions and somewhat lower

spire distinguish *B. cettinensis*. The shells of *B. zeta* (Figs 12–18) are more variable, some of them with high spire (Fig. 15), some other resembling the ones of *B. tentaculata* (Figs 3–6).



Figs 16–22. *Bithynia zeta*: 16–18 – shell, bar equals 2 mm for shells; 19 – male head with penis; 20–22 – penis with tubular penial gland. Scale bar 1 mm for penes





Figs 23–29. *Bithynia cettinensis*: 23–24 – male head with penis; 25–29 – penis with tubular penial gland. Scale bar 1 mm for penes

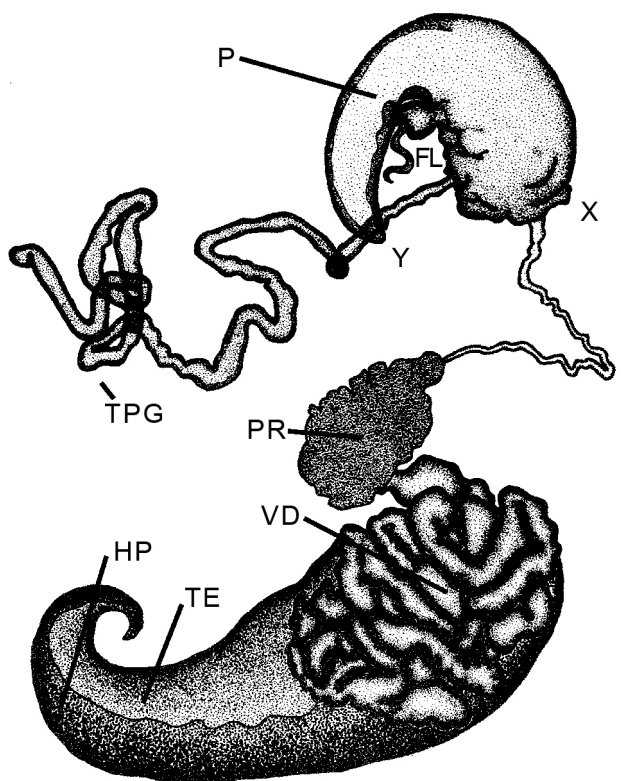


Table 2. Tubular penial gland: penis length ratio in *Bithynia cettinensis* and *Bithynia zeta*

	<i>B. cettinensis</i>	<i>B. zeta</i>
AV	1.33	1.63
SD	0.191	0.244
MAX	1.58	1.95
MIN	1.08	1.36
n	5	5

Fig. 30. Male reproductive organs of *Bithynia tentaculata* (FL – flagellum, left arm of penis harbouring outlet of tubular penial gland; HP – hepatopancreas, digestive gland; P – penis, right arm harbouring vas deferens; PR – prostate; TE – testis; TPG – tubular penial gland; VD – vas deferens; X, Y – points between which the length of right arm, along its right margin, is measured)

The penis is bifid in *B. zeta* (Figs 19–22), *B. cettinensis* (Figs 23–29) and *B. tentaculata* (Fig. 30). Its right arm includes the terminal section of vas deferens. The left arm is known as flagellum, containing the outlet of tubular penial gland situated apically. The penial gland is long and coiled, proximally extending

into the cephalic haemocoel. The mean length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin (from point X to point Y in Fig. 30), is 1.63 for *B. zeta*, and 1.33 for *B. cettinensis* (Table 2).

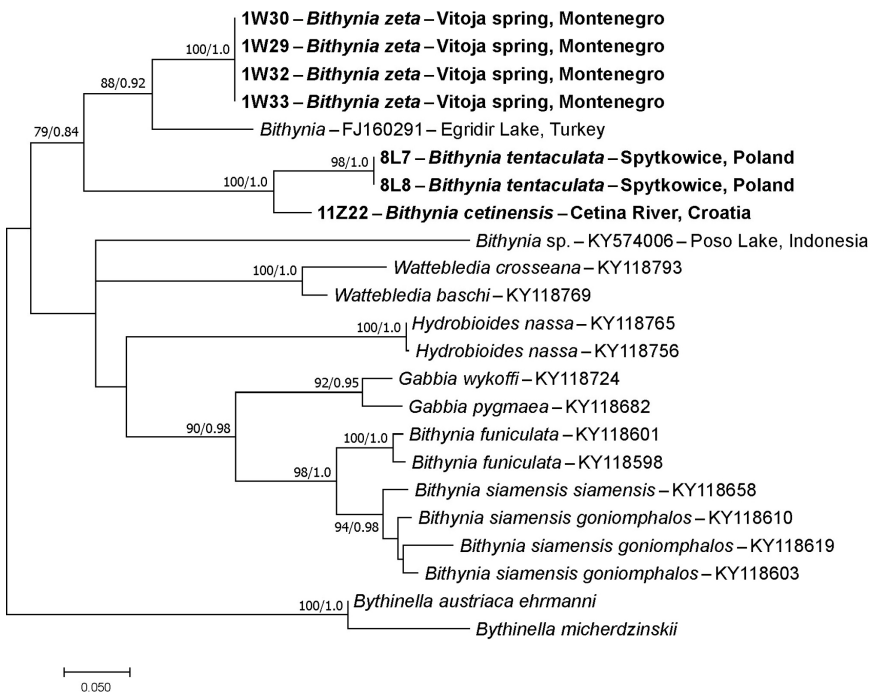


Fig. 31. Maximum likelihood tree based on cytochrome oxidase subunit I (COI); bootstrap supports and Bayesian probabilities given



## MOLECULAR ANALYSIS

In total we analysed seven new sequences of cytochrome oxidase subunit I (COI) (552 bp, GenBank Accession Numbers see Table 1). The tests of XIA et al. (2003) revealed no saturation. The topology of the trees obtained from BI and ML analyses was

identical. The *Bithynia* sequences formed a distinct lineage with four clades (Fig. 31). *B. cettinensis* and *B. tentaculata* were sister clades with p-distance of 0.007. *B. zeta* was more distinct, with p-distance of 0.122 to *B. cettinensis* and 0.154 to *B. tentaculata*. The distances between the three studied species were not smaller than those between the other bithyniid species.

## DISCUSSION

The anatomy of the reproductive organs of *Bithynia* was described by LILLY (1953), FRETTER & GRAHAM (1962), FALNIOWSKI (1989, 1990), and SZAROWSKA (2006). The female reproductive organs are characteristic of the Bithyniidae, but can hardly be used in species distinction (FALNIOWSKI 1990). However, in some publications (e.g. FALNIOWSKI 1989, 1990, GLÖER & PEŠIĆ 2007, GLÖER & BERAN 2009) the term flagellum is used not for the left arm of the penis, but for the tubular penial gland.

FALNIOWSKI (1989, 1990) found that in the Polish *Bithynia* the length ratio of the tubular penial gland (measured along its curvature) to the penis right arm, measured along the curvature of its right margin (from point X to point Y in Fig. 30), was the only anatomical character to allow for species discrimination. The value was 5.0–5.9 for *B. tentaculata* (Linnaeus, 1758), 2.5–3.25 for *B. leachi* (Sheppard, 1823), and ca. 4.3 for *B. troscheli* (Paasch, 1842). Later, this length ratio was considered in species delimitation in *Bithynia* (e.g. GLÖER & YILDIRIM 2006, GLÖER & PEŠIĆ 2007, GLÖER & ROLÁN 2007, GLÖER et al. 2009). For the *Bithynia* from Lake Skadar, the ratio was: 1.5 for *B. zeta*, 2 for *B. skadarskii*, and 4 for *B. radomani*. Considering the photographs in the present

paper, as well as the drawings of FALNIOWSKI (1989, 1990), compared with the ones of GLÖER & YILDIRIM (2006), GLÖER & PEŠIĆ (2007), and GLÖER & ROLÁN (2007), it is clearly visible that the shape and proportions of the penis are more variable, and interspecific differences less marked. The same concerns the tubular gland/penis proportion, less constant in our measurements, but still confirming the determination of our Vitoja spring material as *B. zeta*.

Molecularly *Bithynia zeta* is markedly distinct from the other studied species, and – especially compared with the other sequences of the Bithyniidae from the GenBank – the molecular data confirm its distinctness as a species. On the other hand, the differences between *B. cettinensis* and *B. tentaculata* are not necessarily large enough to confirm the distinctness of those two species. Only somewhat smaller distances can be seen within *B. siamensis* (Lea, 1856), but also between the two nominal species of *Gabbia* Tryon, 1856.

## ACKNOWLEDGMENTS

The study was supported by a grant from the National Science Centre 2017/25/B/NZ8/01372 to Andrzej Falniowski.

## REFERENCES

- BENKE M., BRANDLE M., ALBRECHT C., WILKE T. 2009. Pleistocene phylogeography and phylogenetic concordance in cold-adapted spring snails (*Bythinella* spp.). *Molecular Ecology* 18: 890–903. <https://doi.org/10.1111/j.1365-294X.2008.04073.x>
- CLESSIN S. 1887. Beitrag zur Fauna der Binnen-Mollusken Dalmatiens. *Malakologische Blätter* N. F. pl. ix, p. 62: 43–65.
- EDGAR R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797. <https://doi.org/10.1093/nar/gkh340>
- FALNIOWSKI A. 1989. Przodoskrzelne (Prosobranchia) Polski. I. Neritidae, Viviparidae, Valvatidae, Bithyniidae, Rissoidae, Aciculidae. *Zeszyty Naukowe Uniwersytetu Jagiellońskiego, Prace Zoologiczne* 35: 1–148.
- FALNIOWSKI A. 1990. Anatomical characters and SEM structure of radula and shell in the species-level taxonomy of freshwater prosobranchs (Mollusca: Gastropoda: Prosobranchia): a comparative usefulness study. *Folia Malacologica* 4: 53–142 + 78 tab. fot. <https://doi.org/10.12657/folmal.004.005>
- FALNIOWSKI A., SZAROWSKA M., GLÖER P., PEŠIĆ V., GEORGIEV D., HORSÁK M., SÎRBU I. 2012. Radiation in *Bythinella* (Mollusca: Gastropoda: Rissoidae) in the Balkans. *Folia Malacologica* 20: 1–10. <https://doi.org/10.2478/v10125-012-0006-2>
- FOLMER O., BLACK M., HOEH W., LUTZ R. A., VRIJENHOEK R. C. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299.





- FRETTER V., GRAHAM A. 1962. British prosobranch molluscs. Their functional anatomy and ecology. Ray Society, London.
- GLÖER P., BERAN L. 2009. Redescription of *Bithynia cettinensis* Clessin, 1887 (Gastropoda: Bithyniidae). *Mollusca* 27: 109–111.
- GLÖER P., FALNIOWSKI A., PEŠIĆ V. 2009. The Bithyniidae of Greece (Gastropoda: Bithyniidae). *Journal of Conchology* 40: 1–9.
- GLÖER P., PEŠIĆ V. 2007. The *Bithynia* species from Skadar Lake (Montenegro) (Gastropoda: Bithyniidae). *Mollusca* 25: 7–12.
- GLÖER P., ROLÁN E. 2007. *Bithynia majorcina*, a new species from the Balearics (Gastropoda: Bithyniidae). In: BECKMANN K.-H. (ed.). *Die Land- und Süßwassermollusken der Balearischen Inseln*. ConchBooks, Hackenheim, pp. 159–162.
- GLÖER P., YILDIRIM Z. 2006. Some records of Bithyniidae from Turkey with the description of *Bithynia pesicii* n. sp. (Gastropoda: Bithyniidae). *Malakologische Abhandlungen, Staatliches Museum für Tierkunde Dresden* 24: 37–42.
- HALL T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
- HERSHLER R., PONDER W. F. 1998. A review of morphological characters of hydrobioid snails. *Smithsonian Contributions to Zoology* 600: 1–55. <https://doi.org/10.5479/si.00810282.600>
- KULSANTIWONG J., PRASOPDEE S., RUANGSITTICHAJ J., RUANGJIRACHUPORN W., BOONMARS T., VIYANANT V., PIEROSI P., HEBERT P. D., TESANA S. 2013. DNA barcode identification of freshwater snails in the family Bithyniidae from Thailand. *PLoS ONE* 8: e79144. <https://doi.org/10.1371/journal.pone.0079144>
- LILLY M. M. 1953. The mode of life and the structure and functioning of the reproductive ducts of *Bithynia tentaculata* (L.). *Proceedings of the Malacological Society of London* 30: 87–110.
- MILLER M. A., PFEIFFER W., SCHWARTZ T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop (GCE)*, 14 Nov., New Orleans, LA: 1–8. <https://doi.org/10.1109/GCE.2010.5676129>
- RONQUIST F., TESLENKO M., MARK P. VAN DER, AYRES D., DARLING A., HOHNA S., LARGET B., LIU L., SUCHARD M. A., HUELSENBECK J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539–542. <https://doi.org/10.1093/sysbio/sys029>
- STAMATAKIS A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313. <https://doi.org/10.1093/bioinformatics/btu033>
- SZAROWSKA M. 2006. Molecular phylogeny, systematics and morphological character evolution in the Balkan Rissooidea (Caenogastropoda). *Folia Malacologica* 14: 99–168. <https://doi.org/10.12657/folmal.014.014>
- SZAROWSKA M., HOFMAN S., OSIKOWSKI A., FALNIOWSKI A. 2014. *Heleobia maltzani* (Westerlund, 1886) (Caenogastropoda: Truncatelloidea: Cochliopidae) from Crete and species-level diversity of *Heleobia* Stimpson, 1865 in Europe. *Journal of Natural History* 48: 2487–2500. <https://doi.org/10.1080/00222933.2014.946109>
- TAMURA K., PETERSON D., PETERSON N., STECHER G., NEI M., KUMAR S. 2013. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. *Molecular Biology and Evolution* 28: 2731–2739. <https://doi.org/10.1093/molbev/msr121>
- VAN BOCXLAER B., STRONG E. E., RICHTER R., STELBRINK B., VON RINTELEN T. 2017. Anatomical and genetic data reveal that *Rivularia* Heude, 1890 belongs to Viviparinae (Gastropoda: Viviparidae). *Zoological Journal of the Linnean Society* 182: 1–23. <https://doi.org/10.1093/zoolinnean/zlx014>
- WILKE T., DAVIS G. M. 2000. Intraspecific mitochondrial sequence diversity in *Hydrobia ulvae* and *Hydrobia ventrosa* (Hydrobiidae: Rissoacea: Gastropoda): Do their different life histories affect biogeographic patterns and gene flow? *Biological Journal of the Linnean Society* 70: 89–105. <https://doi.org/10.1006/bjll.1999.0388>
- XIA X. 2000. *Data analysis in molecular biology and evolution*. Kluwer Academic Publishers, Boston, Dordrecht & London.
- XIA X. 2013. DAMBE5: A comprehensive software package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution* 30: 1720–1728. <https://doi.org/10.1093/molbev/mst064>
- XIA X., XIE Z., SALEMI M., CHEN L., WANG Y. 2003. An index of substitution saturation and its application. *Molecular Phylogenetics and Evolution* 26: 1–7. [https://doi.org/10.1016/S1055-7903\(02\)00326-3](https://doi.org/10.1016/S1055-7903(02)00326-3)

Received: March 8th, 2019

Revised: May 7th, 2019

Accepted: May 23rd, 2019

Published on-line: June 12th, 2019

